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Gene Cloning and Biochemical Analysis of Thermostable Chitosanase (TCH-2) from *Bacillus coagulans* CK108

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The DNA sequence of the thermostable chitosanase TCH-2 gene from *Bacillus coagulans* CK108 showed a 843-bp open reading frame that encodes a protein of 280 amino acids with a signal peptide corresponding to 32 kDa in size. The deduced amino acid sequence of the chitosanase from *Bacillus coagulans* CK108 has 61.6%, 48.0%, and 12.6% identities to those from *Bacillus ehemensis*, *Bacillus circulans*, and *Bacillus subtilis*, respectively. C-Terminal homology analysis shows that the enzyme belongs to the Cluster I group. The size of the gene was similar to those from mesophiles of the Cluster I group with regard to higher preference for codons ending in G or C. The recombinant chitosanase was electrophoretically purified to homogeneity by only two steps with column chromatography. The half-life of the enzyme was 40 min at 90°C. The purified protein was also highly stable, retaining above 50% residual activities during treatment with denaturants such as urea (8 M) and guanidine·HCl (4 M) at 37°C for 30 min. The enzyme had a useful reactivity and a high specific activity for producing functional oligosaccharides as well, producing the tetramer as a major product.

Key words: Thermostable chitosanase; Gene cloning; Nucleotide sequencing; *Bacillus coagulans* CK108; Chitosan oligosaccharide

Chitosan is the partly acetylated or nonacetylated counterpart (4-linked 2-amino-2-deoxy-β-D-glucopyranan) of chitin.[1] It is industrially manufactured by chemical deacetylation of chitin from crustacea. Currently, chitosan and its partially degraded oligosaccharides are becoming important because of their potential usefulness and novel application in the field of functional foods, medical aids, pharmaceuticals, and agricultural agents.[2–4] Chitosanases (EC 3.2.1.99), which hydrolyze polymers of (1–4)-β-D-linked-glucosamine (GlcN) residues to chitosan oligomer, have been purified from several microorganisms,[5–10] and most of them catalyze the endo-type cleavage of chitosan with a narrow range of deacetylation degrees.[11–13] The purified chitosanases are classified into at least three groups according to their substrate specificity; the first group hydrolyzes only GlcN-GlcN,[6–7] the second group hydrolyzes GlcNAc-GlcN or GlcN-GlcN,[10–12] and the third group hydrolyzes GlcN-GlcNAc or GlcN-GlcN.[14] Chitosanases cannot in general split the linkage of GlcNAc-GlcNAc. Recently, thermolabile chitosanase genes from several microorganism such as *Bacillus ehemensis* EAG1,[15] *Aspergillus oryzae*,[16] *Streptomyces* sp.,[17] *Norcadioides* sp.,[18] and *Burkholderia gladioli*[19] have been cloned and sequenced. The three-dimensional structure of chitosanases from *Streptomyces* sp. N174[20] and *Bacillus circulans*[21] also have been studied as to catalytic amino acid residues, active cleft, and overall molecular folding. Most of them are thermolabile chitosanases, while little information is available on thermostable chitosanses.

The thermostable chitosanase from *B. coagulans* CK108 hydrolyzes chitosan into 3–6 oligomers by an endo-type catalytic action, and produces no dimer or monomer as a degradation product of the chitosan.[22] However, the mechanism of hydrolysis and the catalytic system involved in the enzyme reaction are still unclear. In this paper, the cloning and nucleotide sequencing of the thermostable chitosanase gene and the characterization of the purified *B. coagulans* CK108 recombinant enzyme are described. We also compared the sequences of bacterial chitosanases, and predicted possible amino acid residues related to catalytic activity and thermostability.

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Abbreviations: TCH-2, thermostable chitosanase from *Bacillus coagulans* CK108; (GlcN), D-glucosamine; (GlcNAc), *N*-acetyl-D-glucosamine; PMSF, phenylmethylsulfonyl fluoride

Materials and Methods

Materials. Chitin, chitosan (100% deacetylated), glycol chitin, and glycol chitosan were purchased from Sigma Co. (St. Louis, USA). Colloidal and soluble chitosan were prepared using the method of Uchida and Ohtakara.[23] Colloidal chitin was prepared by Lockwood's methods.[24] Partially *N*-acetylated chitosan (25–83% acetylated) was prepared from practical grade chitin (Sigma Co.). Commercial chitosanase from *Bacillus* sp. PI-7S was from PIAS Inc. (Osaka, Japan). *Streptomyces* sp. chitosanase was purchased from Sigma Co. Other reagents were of analytical grade.

Bacterial strains, plasmids, and culture conditions. The thermophilic bacterium, *B. coagulans* CK108, was isolated as a potent thermostable chitosanase producer from a hot spring in Korea,[21] and used as the source of chromosomal DNA to clone the enzyme gene. The transformants were screened on CY medium (1.0% glycol chitosan, 0.1% yeast extract, 0.05% tryptone, 0.15% K_2HPO_4, and 0.05% KH_2PO_4; pH 7.0) with or without 2.0% agar, containing appropriate antibiotics (50 μg/ml). The plasmids pUC18, pUC19 (Pharmacia Biotech., Uppsala, Sweden), pBluescript II SK(−) and SK(+) (Stratagene, California, U.S.A.) were used as the cloning vectors. *Escherichia coli* DH5α\{*sup*E44 Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsd*17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1\} was used as the cloning host for recombinant plasmids. *Escherichia coli* BL21(DE3) {*had*S *gal*(λc*Its*857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene1)} was used as the host for pGEX 4T-2 (Pharmacia Biotech., Uppsala, Sweden) to purify chitosanase. All recombinant strains were grown at 37°C on Luria-Bertani(LB) medium containing 50 μg/ml ampicillin for the production of chitosanase.

General recombinant DNA techniques. The DNA was partially digested with *Sau*3AI and electrophoresed on a 1.0% agarose gel. Fragments measuring 4 ~ 10 kb were collected using a Prep-A Gene DNA Purification kit (BioRad, Hercules, U.S.A.). pUC18 was cleaved at the *Bam*HI site and treated with calf intestinal alkaline phosphatase. The *Sau*3AI fragments from the chromosomal DNA were ligated into the dephosphorylated *Bam*HI site of the pUC18. *E. coli* DH5α was transformed with the ligation mixture by electroporation. Transformed cells were grown on a 0.5% glycol chitosan-0.1% Congo red agar medium containing ampicillin (50 μg/ml) at 37°C. The colonies of the enzyme-positive transformants developed clear orange haloes on the red background of the medium.

Analysis of cloned thermostable chitosanase gene. The recombinant plasmid was digested with *Bam*HI and the inserted DNA was isolated using agarose gel electrophoresis. The inserted DNA was used for restriction mapping and subcloning. Various lengths of the DNA fragments of TCH-2 were unidirectionally detected from each side. The deletion mutants of TCH-2 were introduced into *E. coli* DH5α. The chitosanase activity of each transformant was assayed.

Purification of thermostable chitosanase. To construct GST (glutathione *S*-transferase)-chitosanase fusion protein, two oligonucleotide primers, 5′-GGGGATCCATGCGCATCTCCGG-3′ and 5′-GGGAATTCCACTTCATTTGCCA-3′, were synthesized by Takara Biomedicals (Kyoto, Japan). These primers were modified to contain *Bam*HI and *Eco*RI recognition sites to facilitate cloning into the GST fusion protein expression vector, pGEX 4T-2. The PCR product obtained from these primers corresponds to bases 358 to 1200 of the TCH-2 gene. The PCR mixture included 100 pmole primer, 200 ng of template DNA, 20 mM each deoxynucleotide triphosphate, and 1.0 U of *Taq* DNA polymerase in a 50 μl of reaction volume. The 30 rounds of amplification were done with the following cycles: 96°C for 1 min, 72°C for 2 min, and 52°C for 3 min. The amplified DNA was digested with *Bam*HI and *Eco*RI, and cloned into the pGEX 4T-2 digested with *Bam*HI and *Eco*RI. The fusion protein was purified from *E. coli* BL21 (DE3) lysate by affinity chromatography with glutathione-Sepharose 4B (Pharmacia Biotech., Uppsala, Sweden). The purified fusion protein was treated with thrombin for 12 h at room temperature to obtain thermostable chitosanase. The desired protein was purified by HPLC with Protein Pak 300SW semipreparative column (Waters Co., MA, USA) at a flow rate of 0.7 ml/min with 10 mM potassium phosphate buffer (pH 7.5).

Enzyme assay and protein measurement. The reaction mixture containing 250 μl of a 1.0% soluble chitosan, 50 μl of 1.0 M potassium phosphate buffer (pH 6.5), and enzyme solution in a final volume of 1 ml was incubated at 65°C for 30 min with shaking. The reaction was stopped by heating at 100°C for 30 min, followed by centrifugation. The amount of reducing sugar in the supernatant was measured using the modified dinitrosalicyclic acid (DNS) method.[25] One unit of enzyme was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per min. D-Glucosamine was used as a standard. The protein concentration was measured using the Lowry method[26] with bovine serum albumin as a standard.

Analysis of hydrolysis product. The substrate, colloidal chitosan, was dissolved in 10 mM potassium phosphate buffer (pH 6.5) to give 0.5% solution. The

enzyme (0.1 mg protein /ml) was added to 1.0 ml of the substrate solution, and the reaction mixture was incubated at 65°C. After an appropriate reaction time, a portion of the reaction mixture was withdrawn and boilled for 30 min to stop the enzymatic reaction. For analyzing chitosan oligosaccharide with TLC, the supernatants prepared under the conditions as described above were spotted on silica gel plate (Kieselgel 60, Merk, Germany) and developed with *n*-propanol : 30% ammonia water (2:1). Sugars on the TLC plate were stained by spraying 0.1% ninhydrin solution dissolved in 99% ethanol. HPLC was done with an TSK-gel NH_2-60 column (Toso Co., Tokyo, Japan). The products were eluted with an acetonitrile-water mixture (60:40) at a flow rate of 0.8 ml/min and detected with a refractive index (RI) detector. D-Glucosamine, chitosan dimer, trimer, tetramer, pentamer, and hexamer (Seikagaku Co., Tokyo, Japan) were used as an authentic standard. $(GlcN)_n$ product concentrations were calculated from peak areas in the HPLC profiles using the standards curves obtained from the authentic solutions.

DNA sequencing and sequence anlaysis. The plasmid of the subclones was prepared for sequencing using a Wizard Plus SV DNA purification kit (Promega Co., Madison, U.S.A.). Dideoxy DNA sequencing was done with an ALFexpress Autoread sequencing kit (Pharmacia Biotech., Uppsala, Sweden) as specified by the manufacturer. The DNA fragments were analyzed on an ALFexpress Autoread sequencer (Pharmacia Biotech., Uppsala, Sweden). Nucleotide and amino acid sequence analysis, including an open reading frame search and molecular mass calculation, were done using Lagergene software (DNASTAR, Inc., Madison, U.S.A.). Alignment and comparison of the sequences were done with Clustal W. The sequences of bacterial chitosanases were obtained from the GeneBank database.

N-Terminal amino acid sequence analysis. The purified thermostable chitosanase (about 0.1 nmol protein in 10 mM potassium phosphate buffer, pH 7.0) was used for automated Edman degradation with an Applied Biosystem 470A gas-liquid phase protein sequencer. Phenylthiohydantoin (PTH) amino acid derivatives were separated and identified by on-line PTH analyzer model 120A (Applied Biosystems) with a PTH-C_{18} column.

Nucleotide sequence accession number. The nucleotide sequence of *B. coagulans* CK108 thermostable chitosanase gene reported in this article has been assigned the Genebank accession number AF241172.

Results and Discussion

Cloning and nucleotide sequencing of the thermostable chitosanase TCH-2 gene

Recombinant *E. coli* DH5α containing the clone of the *B. coagulans* CK108 genomic DNA was screened for the cloning of the chitosanase gene. A transformant with the chitosanase gene was selected by the forming of an orange halo on the glycol chitosan-Congo red agar medium containing ampicillin. From around 8,000 ampicillin-resistant colonies, two colonies had orange haloes, which suggested that the cloned fragments were derived from the chitosanase gene of *B. coagulans* CK108. To locate the chitosanase gene in the 4.2-kb insert DNA, a series of deletion mutants of pTCH2 was constructed and the chitosanase activity was assayed. Deleting a 2.9-kb region from right to left did not affect the expression of the chitosanase activity. In contrast, deletion of 1.3-kb from left to right caused the loss of chitosanase activity. Consequently, the 1.3-kb *Bam*HI- EcoRI fragment was identified as the region necessary for the production of chitosanase (Fig. 1). To better understand the mechanism of thermostability and substrate specificity, a gene coding for TCH-2 from *B. coagulans* CK108 was sequenced and characterized in the complete nucleotide sequence. The nucleotide sequence of the thermostable chitosanase TCH-2 gene revealed an open reading frame of 843 nucleotides starting with initiation codon ATG and ending with the termination codon TGA at position 1200. The ATG codon was chosen as the translation initiation site because its location was close to a possible ribosome binding site. There is a 5-base se-

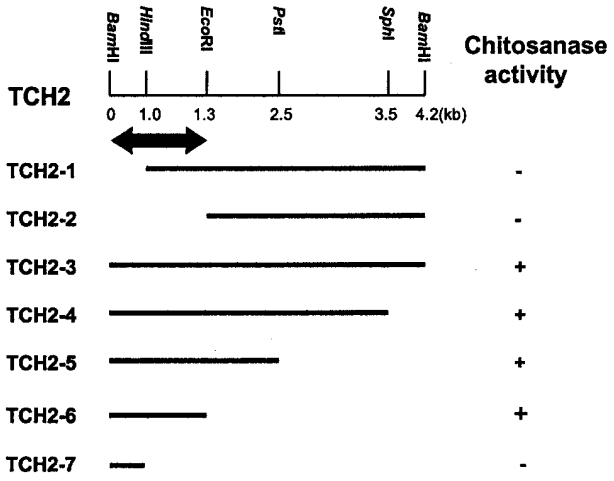

Fig. 1. Restriction Map of the Cloned Gene and Deletion Analysis of 4.2-kb *Bam*HI-*Bam*HI Fragment of pTCH2.

The transformants carrying the plasmids with appropriate deletions were transferred to a LB agar plate containing 0.5% glycol chitosan, 0.1% Congo red, and 50 μg of ampicillin per ml. The thick arrow indicates the necessary region for the expression of chitosanase. +, chitosanase activity detected; −, chitosanase activity not detected.

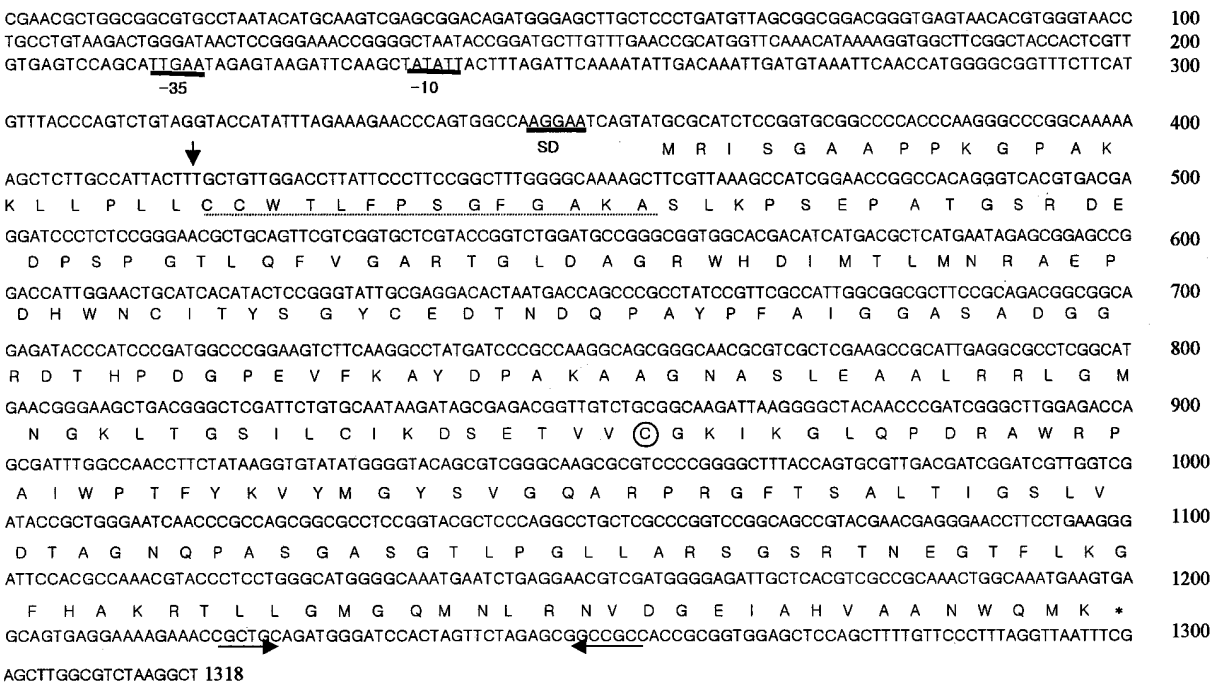

Fig. 2. Nucleotide Sequence of the TCH-2 Gene and Deduced Amino Acid Sequence of the Gene Product.

Coding region starts at position 358 and ends at position 1200. The −35 and −10 region of a putative promoter sequence and a possible Shine-Dalgarno (SD) sequence for the ribosome binding site are underlined. In the 3′-flanking region from the coding sequence, a sequence capable of forming a stem and loop structure, which may be involved in termination, is indicated by arrows. The N-terminal amino acids analyzed by Edman degradation are also underlined with a broken line. The asterisk(*) represents the stop codon. Cysteine residues, presumptive site of thermostability, are circled.

quence, 5′-AAGGA-3′, six bases upstream from the ATG codon that is considerably complementary with the 3′ end of 16S rRNA. The upstream region contains a putative promoter that displays some sequence similarity to the *E. coli* promoter TATAAT (−10) and TTGAA (−35) consensus sequence. Downstream from the TAA stop codon is a G+C rich region of dyad symmetry, capable of forming a stem and loop structure. However, the sequence is not followed by a stretch of T residues, unlike the *E. coli* ρ-independent transcription terminators. In the deduced amino acid sequence, the *N*-terminus of mature chitosanase was located at the 20th amino acid, suggesting that the first 20 amino acids constitute a signal peptide for secretion (Fig. 2).

The G+C content of the coding region for the thermostable chitosanase is 60.4 mol%. This value is higher than the chitosanase genes from the mesophile *B. subtilis* (48.8%), *B. ehemensis* (50.6%), and *B. circulans* (48.6%). In particular, the *Bacillus* coagulans CK108 gene shows a high preference for G or C residues at the third base (the wobble position) of the codons; the G+C content at that position is 71.4 mol %, whereas it is 42.6 mol% in the *B. subtilis* gene (data not shown). Changes from A/T to G/C in DNA sequence, particularly in the wobble position of each codon, are thought to be one of the mechanism of gene stabilization at high temperatures.[27]

Comparison of deduced amino acid sequence of the chitosanase TCH-2 gene with those of other chitosanases

The deduced TCH-2 amino acid sequence was compared with the sequences of nine bacterial chitosanases (Fig. 3). The ten sequences were linearly aligned by the clustal method. The TCH-2 sequence showed identity of 61.6%, 48.0%, 12.6%, 12.5%, and 10.8% to the sequences of *Bacillus ehemensis, Bacillus circulans, Bacillus subtilis, Streptomyces* sp. N174, and *Nocardioides* sp. N106 chitosanases, respectively. Linear alignment of two sequences, TCH-2 and *B. ehemensis* chitosanase, showed marked similarity between the two enzymes. The overall sequence similarity was calculated as 61.6%, which is high for the degree of sequence similarity between thermostable and thermolabile enzymes in interspecies, strongly suggesting that the two chitosanases may have similar three-dimensional structures. The C-terminal sequence similarities among the chitosanases are calculated as 96% (*Bacillus circulans: Bacillus ehemensis: Bacillus coagulans* CK108), 93% (*Bacillus* sp. CK4 : *Bacillus subtilis*), 89% (*Sphingobacterium multivorum: Matsuebacter chitosanotabidus*), and 95% (*Streptomyces* sp. N174: *Nocardioides* sp. N106: *Amycolatopsis* sp.). The 96% similarity between *Bacillus ehemensis* and *Bacillus coagulans* CK108 indicates that they belong to the same group, that is, cluster I (Fig. 4). Although

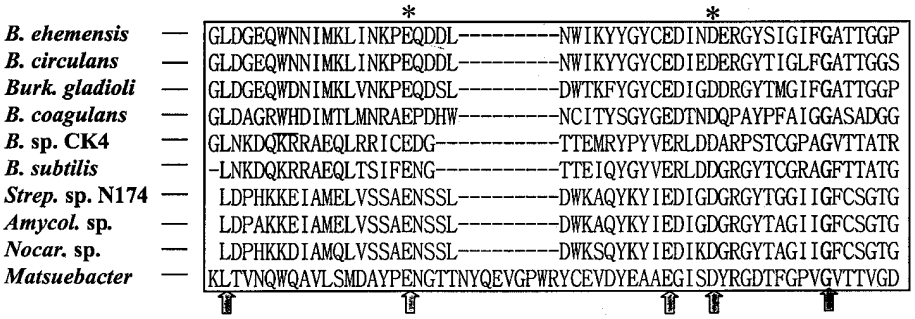

Fig. 3. Alignment of Putative Catalytic N-Terminal Segment.

Sequence segments shown here are from *Bacillus ehimensis* (GenBank accession number: AB008788), *Bacillus circulans* (D10624), *Burkholderia gladioli* (AB029336), *Bacillus coagulans* CK108 ($AF_2$41172), *Bacillus* sp. CK4 (AF165188), *Bacillus subtilis* (Z99117), *Streptomyces* sp. N174 (L07779), *Amycolatopsis* sp. CsO-2 (AB041775), *Nocardioides* sp. strain N106 (L40408), *Matsuebacter chitosanotabidus* (AB006851), and *Sphingobacterium multivorum* (AB030253). *, essential catalytic residues of *Streptomyces* sp. N174; the arrows, the target amino acid residues in TCH-2 for site-directed mutagenesis. The homology search was done with Lasergene software (DNASTAR Inc.).

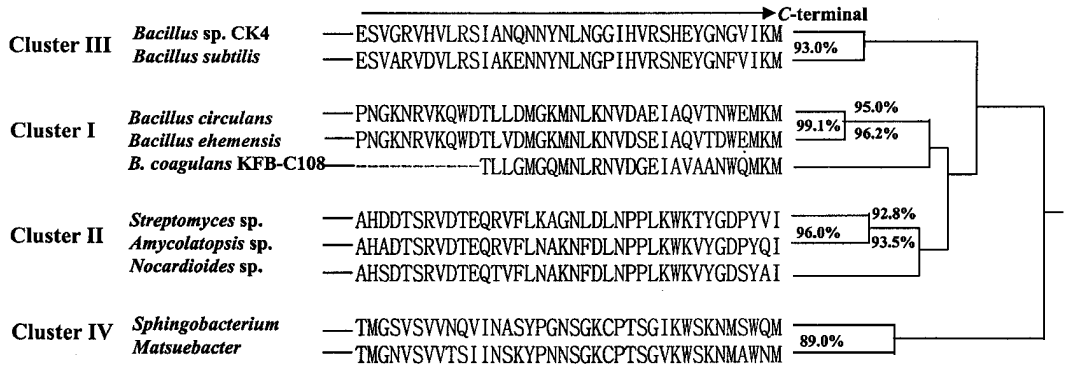

Fig. 4. Amino Acid Sequence Alignment of C-Terminal Region.

The origin and accession numbers of the sequences are as follows: *Bacillus* sp. CK4, AF160195; *Bacillus ehemensis*, AB008788; *Bacillus subtilis*, Z99117; *Bacillus circulans*, D10624; *Amylocolatopsis* sp. CsO-2, AB041775; *Streptomyces* sp., L07779; *Matsuebacter chitosanotabidus*, AB006851; *Sphingobacterium multivorum* csoA, AL109849; *Nocardiodes* sp., L40408. The similarity search were done with Lasergene software (DNASTAR Inc.).

some similarities of amino acid sequences were found in N-terminal segments (62–98 of the *Bacillus coagulans* CK108 sequence), the chitosanase has no extensive similarity with other groups of chitosanase in other parts. The N-terminal segments of these bacterial chitosanases have a conserved five amino acid residues, which are thought to be putative catalytic sites of chitosanase.

The chitosanase from *Streptomyces* sp. N174 is the first enzyme for which an atomic resolution X-ray structure was published.[20] In the study, Glu-22 acts as a general acid catalyst that donates a proton to the glycosylic oxygen, and Asp-40 contributes to the lowering of the energy barrier of the reaction by stabilizing the transient carbonium ion intermediate electrostatically. The mechanism was confirmed by site-directed mutagenesis of the proposed catalytic residues.[28] Studies on the three-dimensional structure of *B. circulans* chitosanase showed that the conserved N-terminal regions containing Leu-64, Glu-94, and Asp-98 were located in the active cleft, and the overall molecular folding is similar to a chitosanase from *Streptomyces* sp. N174.[22] Furthermore, in most glycosyl hydrolases, the catalytic amino acids are aspartate or glutamate residues conserved in the region sharing amino acid sequence similarities. The catalytic role of residues Asp-98 and Leu-64 (Fig. 3) are identified by some recently-obtained data. In this site-directed mutagenesis experiment, Leu-64 was proposed as a catalytic residue corroborating the conclusion drawn from this work.[10] On the other hand, glutamate and aspartate residues do not play an important role in catalysis, seemingly essential for bacterial chitosanases.

Several structural explanations have been considered for the increase in the thermostability of a protein, such as increases of hydrophobicity in the interior of a molecule, helix stability, and tight packing interactions inside the molecules.[29] Because the crystal structure of *B. coagulans* CK108 is not available, a detailed discussion is not possible. However, the proteins from thermophilic bacteria are generally

thermostable, and the information about factors that contribute to the thermostability of proteins is thought to involve their amino acid sequences, and can be partially obtained by comparing these amino acid sequences with those of thermolabile proteins.[30] The only significant difference between the two chitosanases from *B. coagulans* CK108 and *B. ehemensis* is thermostability. The thermolabile chitosanase from *B. ehemensis* and *B. circulans*, and the thermostable chitosanase from *B. coagulans* CK108 are similar in primary structure. Linear alignment of amino acid sequences showed a marked similarity among three enzymes; *B. ehemensis: B. coagulans* CK108 (61.6% similarity), *B. circulans: B. coagulans* CK108 (48.0% similarity), and *B. ehemensis: B. circulans* (84.1% similarity). The overall sequence similarity is quite high for the degree of interspecies sequence homology. The *B. ehemensis* and *B. circulans* chitosanases contain in common three cysteinyl residues at position 21, 93, and 167. It is interesting to note that the thermostable enzyme from *B. coagulans* CK108 contains one additional cysteinyl residue at position 156 besides three residues located at position 21, 93, and 167 equivalent to those in the enzymes from *B. ehemensis* and *B. circulans* (Fig. 2). This contrasts with the lack of cysteine in the *B. stearothermophilus* enzyme, which has very high thermostability.[31] Because free cysteines occurring in the exterior of proteins are a potential function of thermal instability, Cys-156 of the *B. coagulans* CK108 enzyme may occur in the interior of the protein, with a positive effect on the thermostability. To find whether these cysteine residues have some role in the thermostability of enzyme, site-directed mutagenesis of these residues and analysis of resulting alterations in the conformational stability are under way.

Purification of chitosanase from GST-chitosanase fusion protein

The chitosanase TCH-2 was simply purified by only two steps consisting of glutathione affinity chromatography and a semipreparative HPLC column. A substantial portion of the GST-chitosanase fusion protein ($Mr = 65{,}000$ Da) was in the insoluble pellet as inclusion bodies. To maximize the yield of the soluble chitosanase, we examined the enzyme induction conditions, and found that the enzyme induction with 0.1 mM IPTG at 25°C for 20 hr produced the maximum level of soluble active fusion protein. Thus, we routinely used these conditions. In the first purification step, thrombin cleavage and elution of the full length chitosanase from glutathione-Sepharose beads gave one major band of 32,000 ± 2,000 Da and several minor bands. To remove the minor bands, thrombin-eluted chitosanase was put on a semipreparative HPLC column at the second step. The enzyme, purified 80-fold with a 16% overall yield, appeared to be homogeneous by the criteria of polyacrylamide gel electrophoresis (Fig. 5). The molecular mass of the protein is approximately 32,000 ± 2,000 Da based on its motility calculated by TSK-Gel HW-55F gel permeation chromatography (data not shown).

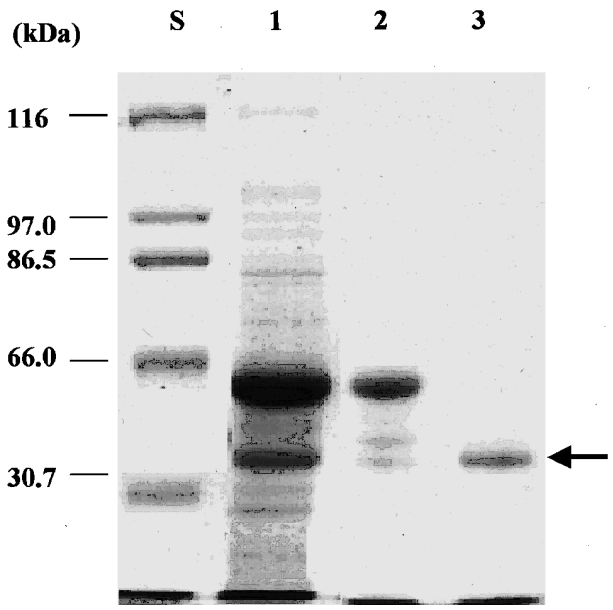

Fig. 5. Purification of Chitosanase from GST-Chitosanase Fusion Protein.

Lane 1, Crude enzyme; lane 2, GST-chitosanase following fusion protein adsorption; lane 3, semiprepative HPLC following thrombin elution; lane S, size marker.

Stability of chitosanase

The optimal temperature for chitosanase activity was 65°C and the optimum pH was 6.5 under the standard assay conditions. The thermostability of the recombinant protein was examined by measuring the residual activity after incubation at various temperatures. The residual activities after heat treatment of the enzyme (0.5 mg protein per ml of 50 mM potassium phosphate buffer, pH 6.5) at 80°C for 30 and 60 min were 90 and 73%, respectively. Although the enzyme activity was completely lost after 30 min at 100°C, the enzyme retained its full activity after incubation at 60°C for 60 min, and 64% of the initial activity remained even after heat treatment at 90°C for 30 min (Fig. 6A). We found that the enzyme is stable at high concentrations of chemical denaturants such as ethanol and SDS. The enzyme was not inactivated when incubated with 50% ethanol at 37°C, and retained about 72% of its activity after incubation with 5% SDS at 37°C for 1 h (data not shown). The enzyme also retained its full activity after incubation with 4 M urea or 2 M guanidine HCl at 37°C for 30 min (Fig. 6B). It is noteworthy that the enzyme is quite stable even in 8 M urea, which causes complete denaturation of most proteins. The optimum temper-

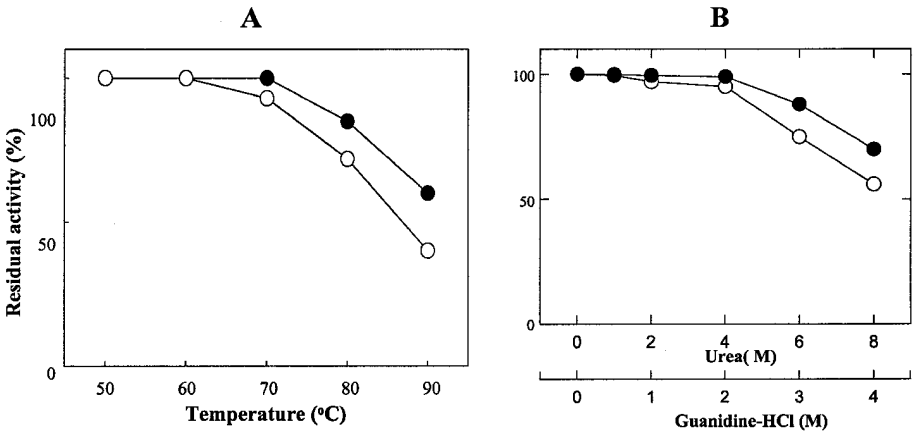

Fig. 6. Effects of Temperature and Protein Denaturants on the Stability of Chitosanase.
(A) After the enzyme was incubated at 50, 60, 70, 80, and 90°C for 30 (●) and 60 min (○), residual activities were measured to find the degree of thermostability of the enzyme. (B) The enzyme (0.5 mg/ml) was incubated in 10 mM potassium phosphate buffer (pH 7.5) containing urea (●) and guanidine HCl (○) at 37°C for 30 min, and then the residual activity was assayed after removal of the denaturants by ultrafiltration with Centricon-10.

ature (65°C) for the recombinant chitosanase activity is in the range of the normal optimum temperature of thermostable enzymes (60–100°C) and the purified chitosanase shows a high stability in this temperature range compared to the other bacterial chitosanases. Furthermore, the stability of *B. coagulans* CK108 chitosanase to heat inactivation and the effects of temperature on its activity confirm its thermostable nature.

Substrate specificity

The activities of the purified chitosanase upon chitosan, chitosan derivatives, and other polysaccharides are presented in Table 1. Colloidal chitosan, soluble chitosan, and glycol chitosan were found to be good substrates. The *K*m for colloidal chitosan and soluble chitosan were 1.0 and 1.7 mg/ml, and the *V*max were 611 and 516 unit/mg, respectively. Colloidal chitosan was hydrolyzed 3.6 times faster than glycol chitosan. The enzyme was specific for chitosan, causing no cleavage of chitin, cellulose, amylose, or starch. Chitosanases can be classified into at least three groups according to their cleavage specificity as described previously in this paper. The purified enzyme belongs to the first group, which is able to hydrolyze only chitosan. The chitosanases classified in the group hydrolyzing only chitosan, also can catalyze colloidal chitin and partially that of *O*-hydroxyethylated chitosan as well.[6-7] This new enzyme is distinct from other enzymes in the group on the substrate specificity of colloidal chitin non-degradation. To examine the substrate specificity of the chitosanase on chitosan with different degrees of deacetylation (DDA), the various substrates were prepared by different procedure for *N*-acetylation. The relative activity increased when DDA of chitosan grew. This indicates that the physical form and DDA of substrate affects the rate of hydrolysis. However,

Table 1. Substrate Specificity of Thermostable Chitosanase TCH-2 from *Bacillus coagulans* CK108

Substrate (1.0%)	Relative[a] activity (%)	*V*max (unit/mg)	*K*m (mM)	k_{cat}/Km ($mM^{-1}S^{-1}$)
Chitin[b]	0	0	—	—
Colloidal chitin	0	0	—	—
Glycol chitin	0	0	—	—
Soluble chitosan	83.1	516	1.7	8.47
Colloidal chitosan	100	611	1.0	17.5
Glycol chitosan	27.8	171	20.9	0.26

[a] The relative activity was expressed as percentage of the activity measured with soluble chitosan.
[b] The purified chitin was purchased from Sigma Co.

no great difference was found in the hydrolysate of soluble chitosan and colloidal chitosan with different DDA (data not shown). The *K*m and k_{cat} of the enzyme were also temperature-dependent; their values increased as the reaction temperature increased.[29] At 65°C, the *K*m for colloidal chitosan and soluble chitosan were 1.3- to 1.8-fold higher than those at 30°C. Similarily, the k_{cat} values were 2.0- to 2.8-fold higher at 65°C than at 30°C (Table 2). Distinguishing features of a truly thermostable enzyme are that, compared to their mesophilic counterparts, they are more stable against heat denaturation and have low activity at ambient temperatures and high activity at higher temperatures.[32]

Analysis of hydrolysis products

The catalytic pattern of the chitosanase TCH-2 was examined using colloidal chitosan as the substrate. A change of hydrolysis products from colloidal chitosan was observed during incubation with the recombinant purified enzyme at 65°C for 12 h. Initially, colloidal chitosan was hydrolyzed to

Table 2. Effects of Temperature on Enzyme Reaction Kinetics

Substrate (1.0%)	30°C				65°C			
	Relative activity[a] (%)	*V*max (U/mg)	*K*m (mM)	k_{cat} (s^{-1})	Relative activity[a] (%)	*V*max (U/mg)	*K*m (mM)	k_{cat} (s^{-1})
Soluble chitosan DDA[b] 99%	59.4	365	1.3	6.3	83.1	510	1.7	14.4
Soluble chitosan DDA 83%	55.4	340	2.1	6.1	72.0	442	3.2	12.3
Soluble chitosan DDA 71%	33.8	208	2.6	3.0	44.0	270	4.4	7.6
Soluble chitosan DDA 53%	8.9	55.1	4.3	0.9	13.1	80.5	6.8	2.3
Colloidal chitosan DDA 99%	91.1	559	0.7	7.2	100	614	1.0	17.3
Colloidal chitosan DDA 83%	78.9	484	3.4	6.1	87.1	535	5.8	15.1
Colloidal chitosan DDA 71%	39.4	242	2.1	5.5	67.0	411	3.7	11.6
Colloidal chitosan DDA 53%	21.1	130	0.3	2.0	31.7	197	0.5	5.5

[a] The relative activity was expressed as a percentage of the activity measured with soluble chitosan DDA 99% at 65°C.
[b] Degree of deacetylation was calculated using the colloidal titration method.

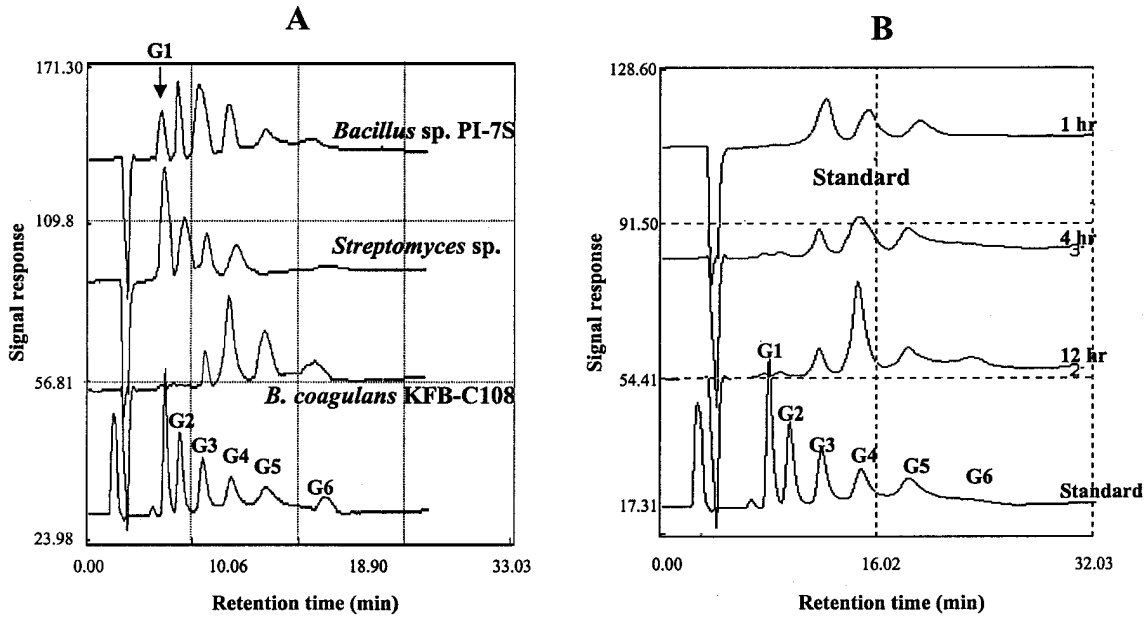

Fig. 7. HPLC Profiles of the Chitosan Oligosaccharide Produced by Thermostable Chitosanase TCH-2 from *Bacillus coagulans* CK108. The enzyme (0.1 mg/ml) was added to 0.5 ml of 1% colloidal chitosan dissolved in 10 mM potassium phosphate buffer, pH 6.5. The products were analyzed on a TSK-Gel $NH_2$60 column for the chitosan oligosaccharide. Standard G1–G6 indicate standard $(GlcN)_n$ (n = 1–6). The reaction was done at 65°C.

$(GlcN)_3 \sim (GlcN)_5$ (80% of total products) and negligible dimer. After 12 h of incubation, tetramer was increased to about 30% in the hydrolysate, and trimer and pentamer were decreased, but yet no monomer and dimer (Fig. 7). In the hydrolysis products of colloidal chitosan, (GlcN)4 was detected as the major product with small amounts of trimer, pentamer, and hexamer, but with no monomer and dimer (Fig. 7). It suggested that the mode of action of the enzyme is an endo-type. Endo-type chitosanases have been reported from several microorganisms and their degrading patterns on chitosan were similar.[5,8–10] Although the amount of oligomers were variable in each case, these enzymes hydrolyzed chitosan into 1 ~ 6 oligomers by an endo-type catalytic action. The thermostable chitosanase TCH-2 produces functional oligomers, trimer ~ hexamer, with a high rate about 90% of total yield under 65°C for 12 h. The reaction pattern of this chitosanase with its thermostability makes the enzyme as a good candidate for the biotechnological applications in the industrial production of functional chitooligosaccharide.

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